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Membrane Hyperpolarization by Sperm-Activating and -Attracting Factor Increases cAMP Level and Activates Sperm Motility in the Ascidian *Ciona intestinalis*

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In the ascidian *Ciona intestinalis* (and *C. savignyi*), sperm-activating and -attracting factor (SAAF) is released from the egg at fertilization and stimulates both Ca^{2+} influx and a transient increase in cAMP level of the sperm, leading to the activation of sperm motility (M. Yoshida *et al.*, 1994, *Dev. Growth Differ.* 36, 589–595). In this paper we show in *C. intestinalis* that valinomycin, a potassium-selective ionophore, as well as SAAF, activated sperm motility, and this activation was suppressed by extracellular high K^+. Membrane potential measurements showed that both SAAF and valinomycin increase K^+ permeability of sperm and induce membrane hyperpolarization, the amplitude of which depends on the external K^+ concentration. The membrane potential and intracellular K^+ concentration of *Ciona* sperm without SAAF were estimated to be about −50 mV and 560 ± 40 mM, respectively. After treatment with SAAF or valinomycin the membrane potential became almost equal to the equilibrium potential of K^+ (−100 mV), and the cAMP level increased in artificial seawater. A potent voltage-dependent K^+ channel blocker, MCD peptide, at the concentration of 10 μM blocked SAAF-induced hyperpolarization of the cells, increase in cAMP, and sperm motility. These results suggest that membrane hyperpolarization produced by the opening of K^+ channels elevates cAMP synthesis and leads to the activation of sperm motility in *Ciona*.

Key Words: **ascidian; sperm motility; potassium efflux; membrane hyperpolarization; adenylyl cyclase.**

INTRODUCTION

Cyclic AMP and calcium are widely known to be the critical factors regulating motility and acrosome reaction in the sperm of vertebrate and invertebrate species (Tash and Means, 1983; Morisawa, 1994; Schackmann, 1989; Ward and Kopf, 1993). The cAMP increased by the effect of speract or resact derived from sea urchin egg triggers the activation of sperm motility (Hansbrough and Garbers, 1981; Suzuki and Garbers, 1984) or chemotaxis (Ward *et al.*, 1985), respectively, through the activation of protein kinase and the subsequent protein phosphorylation (Ward and Kopf, 1993). The role of protein phosphorylation in the regulation of sperm motility in sea urchin sperm was also emphasized by Bracho *et al.* (1998). In the salmonid fish, tyrosine phosphorylation of proteins through a cAMP-dependent process initiates sperm motility (Hayashi *et al.*, 1987). The activation of mammalian sperm also requires cAMP (Morton *et al.*, 1974) and protein phosphorylation (Brandt and Hoskins, 1980; Tash, 1990). In ascidians, cAMP-dependent initiation of sperm motility (Brokaw, 1982; Opresko and Brokaw, 1983; Morisawa *et al.*, 1984) occurs through phosphorylation of some proteins (Dey and Brokaw, 1991; Chaudhry *et al.*, 1995). Although the intracellular cell signaling mechanisms in these phenomena have been well investigated in these animals, the trans-

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membrane cell signals that lead to cAMP synthesis are unknown with regard to the initiation and activation of sperm motility.

Schultz *et al.* (1992) have shown in *Paramecium* that the hyperpolarization of the plasma membrane by a decrease in external K^+ concentration induces cAMP synthesis through the activation of adenylyl cyclase. The elevation of cAMP level was blocked by K^+ channel blockers, suggesting that changes in the membrane potential directly regulate adenylyl cyclase activity. With regard to the mechanisms regulating the sperm acrosome reaction, on the other hand, it has been suggested that potential changes of sperm plasma membrane regulate adenylyl cyclase activity (Beltran *et al.*, 1996) and Ca^{2+} level (González-Martínez and Darszon, 1987; González-Martínez *et al.*, 1992) in sea urchin sperm. It has also been proposed that a decrease in external K^+ concentration surrounding trout sperm causes membrane hyperpolarization (Tanimoto and Morisawa, 1988; Gatti *et al.*, 1990; Boitano and Omoto, 1991; Tanimoto *et al.*, 1994) and triggers a cAMP-dependent initiation of sperm motility (Morisawa and Ishida, 1987).

Recently, Yoshida *et al.* (1994) found sperm-activating and -attracting factor (SAAF) in egg seawater in the ascidians *Ciona intestinalis* and *C. savignyi*. This finding opened up new insights into the investigation of cell signaling for the regulation of sperm motility. This factor is released from the egg at fertilization and stimulates both Ca^{2+} influx and a transient cAMP increase, leading to the activation of sperm motility. It has also been revealed that the elevation of cAMP by a phosphodiesterase inhibitor, theophylline, causes the activation of *Ciona* sperm motility even in the absence of extracellular Ca^{2+}, but theophylline does not elicit the attracting action of sperm toward the factor, suggesting that cAMP increase induces the activation of sperm motility but is not sufficient for sperm attraction.

Here we measured membrane potential changes of sperm plasma membrane, intracellular K^+ concentrations, and cAMP levels during SAAF-dependent activation of sperm motility. The results suggest that membrane hyperpolarization through the opening of K^+ channels increases cAMP synthesis and leads to the activation of sperm motility in *Ciona*.

MATERIALS AND METHODS

$DisC_3(5)$, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 3-isobutyl-1-methylxanthine (IBMX), valinomycin (Val), nigericin, and theophylline (TP) were purchased from Sigma Chemical Co. (St. Louis, MO); tetraethylammonium chloride (TEA) and cremophor EL were from Nacalai tesque (Kyoto, Japan); 4-aminopyridine (4-AP) was from Wako Pure Chemical Industries, Ltd. (Oosaka, Japan); the AM esters of BCECF were from Dojindo Laboratories (Tokyo, Japan); the AM esters of PBFI were from Molecular Probes, Inc. (Eugene, OR); and mast cell-degranulating peptide (MCD peptide) was from Alamone Labs, Ltd. (Jerusalem, Israel).

Artificial seawater (ASW) consisted of 462 mM NaCl, 9 mM KCl, 10 mM $CaCl_2$, 48 mM $MgCl_2$, 10 mM Hepes, pH 7.8. NaCl was substituted for KCl ($[Na^+ + K^+]$ 482 mM) in the ASW with different K^+ concentrations. $CaCl_2$ was substituted for NaCl in the Ca^{2+}-free ASW (CaFASW).

The specimens of the ascidian *C. intestinalis* were collected at Onagawa Bay, Miyagi prefecture, and Yokohama Bay, Kanagawa prefecture. They were kept in an aquarium under constant light to prevent spontaneous spawning. Semen (dry sperm) was obtained by excising the sperm duct and was kept on ice or in a refrigerator (4°C) until use. SAAF was partially purified from the egg seawater of *C. intestinalis* by the method described previously (Yoshida *et al.*, 1994).

Assessment of Sperm Motility

Semen was diluted 1000 times (6×10^7 cells/ml) in ASW or ASW with different K^+ concentrations, and 20 μl of sperm suspension was put on a glass slide and placed under a microscope (Nikon Optiphoto, Tokyo, Japan) which was connected to a video camera (Hamamatsu C2400 SIT; Shizuoka, Japan). Two microliters of SAAF or Val (50 nM final concentration) was then added to the suspension, and the percentage of motile sperms was assessed manually from video records. The concentration of a solvent, DMSO, was kept below 1% v/v in all experiments.

Measurement of Membrane Potential

Semen was diluted 2000 times in 1 ml (3×10^7 cells/ml) of experimental medium in a cuvette, and $DisC_3(5)$ (0.5 μM final concentration) was added to the suspension. The mitochondrial potential was dissipated by the addition of 1 μM CCCP, and the fluorescence was then monitored with a fluorescence spectrophotometer (Hitachi 650-10S; Tokyo, Japan) at 620/670-nm excitation/emission wavelength pair (Babcock *et al.*, 1992; Zeng *et al.*, 1995). The hyperpolarization of the plasma membrane decreases the fluorescence intensity under this condition. The fluorescence was converted to the membrane potential (V_m) as follows. First, $[K^+]_{ex}$ was changed by adding various volumes of 2 M KCl solution in the presence of Val, and the fluorescence was measured. The V_m was calculated with the Nernst equation, assuming the intracellular potassium concentration of 560 mM and that the membrane is K^+ electrode under the influence of the K^+-selective ionophore Val (Gisin *et al.*, 1978). The V_m under various experimental conditions were calculated from the fluorescence intensity using this calibration curve.

Measurement of Intracellular pH

Intracellular pH (pH_{in}) was determined with BCECF using the ratio method (Balkay *et al.*, 1992; Márián *et al.*, 1997). Semen was diluted in 10 volumes of CaFASW containing a detergent, cremophor EL, at the concentration of 0.025% (stock solution 25% in DMSO) and 10 μM BCECF-AM (stock solution 1 mM in DMSO) and was incubated for 2 h by slowly shaking at 18°C in the dark. The sperm suspension was then added to 50 volumes of CaFASW, mixed, and centrifuged at 1200*g* for 10 min. The pellet was suspended again in 10 volumes of CaFASW and incubated for 1 h in the same manner to allow hydrolysis of the AM esters of the dye. The BCECF-loaded sperm were washed by centrifugation and suspended again in 10 volumes of CaFASW. The 20- to 40-μl sperm suspension was diluted with ASW of different pH in the cuvette, and the fluorescence was monitored at 18°C at 490/535- and 440/535-nm excitation/emission wavelength pairs by a fluo-

rescence spectrophotometer (Hitachi F-2000; Tokyo, Japan). The intracellular pH was calibrated with 50 nM free BCECF in ASW solutions of different pH at the range of 5.6–8.5 and calculated by the following formula: pH_{in} = ([fluorescence ratio 490 nm/440 nm] + 18.594)/3.487.

Calibration of Intracellular K^+ Concentration

Sperm were loaded with a pH-sensitive fluorescence dye, BCECF-AM, as described above, and the loaded sperm were incubated in ASW with different K^+ concentration ([$Li^+ + K^+$] 482 mM). Nigericin was added to the suspension and the shift of pH_{in} was monitored at 490/535- and 440/535-nm excitation/emission wavelength pairs. As a control, digitonin was added to make pH_{in} equivalent to extracellular pH (pH_{ex}). Intracellular K^+ concentration was calculated by the methods described previously (Babcock *et al.*,1992; Balkay *et al.*, 1997).

Monitoring of Intracellular Potassium Concentration Changes

Sperm was loaded with 10 μM PBFI-AM, a fluorescence K^+ indicator dye, as well as other indicators. The change of intracellular K^+ concentration was monitored by fluorescence at 344/480-nm excitation (10 nm)/emission (16 nm) wavelength pair.

Assay of cAMP

We used cAMP enzyme-immunoassay system (dual range) from BIOTRAK (PRN 225; Amersham Pharmacia Biotech, England). Semen was diluted 2000 times (3×10^7 cells/ml) in ASW, and the samples were added to the sperm suspension. After the necessary incubation time, each 180 μl of the suspension was mixed with 20 μl of Kit buffer (lysis reagent) to stop cAMP synthesis and to lyse the cells, and each 100 μl of that mixture was put into the wells contained in the Kit to quantify cAMP as described in the manual. Cyclic AMP level of each sample was calculated by measuring 450-nm absorbance with a microplate reader (Model 550; Bio-Rad, Richmond, CA).

Measurement of Ion Concentrations

Sodium, potassium, calcium, and magnesium concentrations were measured by atomic absorption spectrometer (Hitachi 180-50; Tokyo, Japan). Chloride concentration was analyzed by Buchler Digital Chloridometer (Buchler, U.S.A.).

RESULTS

Effects of Valinomycin and Extracellular Potassium on the Activation of Sperm Motility

One nanomolar or higher concentration of Val, a K^+-selective ionophore (Gisin *et al.*, 1978), caused the activation of *Ciona* sperm motility in ASW (Fig. 1), in a manner similar to that of SAAF (Yoshida *et al.*, 1994). The Val- and SAAF-induced initiation of sperm motility was blocked by an increase in $[K^+]_{ex}$ (Fig. 2). ASW containing 22 mM or higher K^+ completely abolished SAAF- and Val-induced sperm motility. The inhibitory effect of high $[K^+]_{ex}$ was

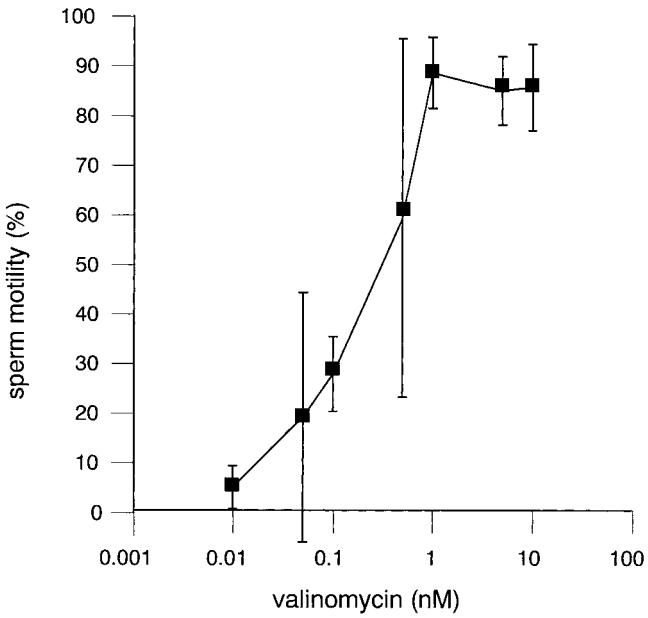

FIG. 1. Valinomycin-induced activation of sperm motility. Sperm were suspended and incubated in ASW ([K^+] 9.39 mM, pH 7.8) for 15 min, and Val dissolved in DMSO was added to the sperm suspension. Sperm motility was measured as described under Materials and Methods. DMSO (1%) had no effect on sperm motility. Data represent means ± SD (n = 4).

reversible; a subsequent decrease in $[K^+]_{ex}$ by dilution with ASW reinitiated sperm motility (data not shown). The effect of Val was not dependent on the presence of external Ca^{2+} (Fig. 2), although SAAF-induced sperm motility required external Ca^{2+} (Yoshida *et al.*, 1994).

MCD peptide, one of the blockers of voltage-dependent K^+ channels (Walter *et al.*, 1989; Reza Zaiai *et al.*, 1990), completely inhibited the SAAF-induced activation of sperm motility at 10 μM (Fig. 3). The Val-induced sperm motility was also completely inhibited by MCD peptide, while sperm motility induced by phosphodiesterase inhibitors (IBMX or TP) was not inhibited by MCD peptide (Fig. 3). None of the other K^+ channel blockers, 4-AP, TEA^+, Cs^+, or Ba^{2+}, inhibited the SAAF-induced activation of sperm motility (data not shown).

Valinomycin- and SAAF-Induced K^+ Efflux and Hyperpolarization of Plasma Membrane

Sperm were loaded with $DisC_3(5)$ in ASW, and the fluorescence intensity was measured under various experimental conditions. The fluorescence was converted to membrane potential as described under Materials and Methods. In the absence of SAAF or Val, the fluorescence was stable, and subsequent additions of 5, 10, and 20 μl of 2 M KCl, which raised the $[K^+]_{ex}$ by 10, 20, and 40 mM K^+, respectively, did not induce any changes in the fluorescence (Fig.

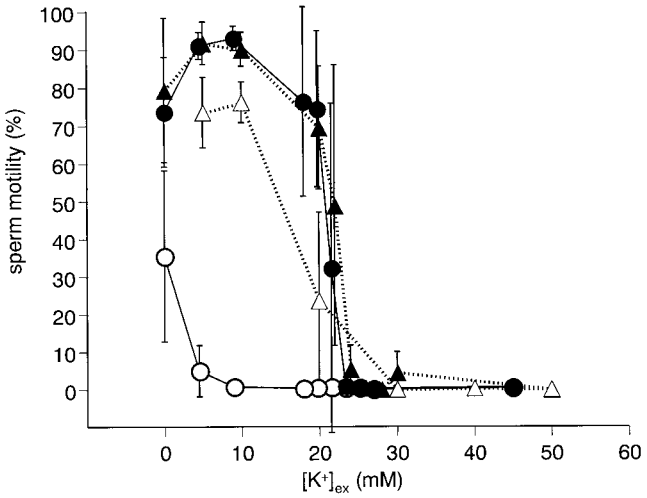

FIG. 2. Effects of $[K^+]_{ex}$ on Val- or SAAF-induced activation of sperm motility. Sperm were suspended and incubated for 15 min in ASW containing different concentrations of K^+ (open circle). Spontaneous activation of sperm motility is only slightly observed at 0–5 mM $[K^+]_{ex}$. The sperm motility was measured after addition of SAAF (filled circle) or 50 nM Val (filled triangle). Val also activated sperm motility in CaFASW (open triangle). Data represent means ± SD (n = 4).

4A). These results suggest that the membrane potential of the resting (immotile) sperm is less responsive to an increase in $[K^+]_{ex}$, i.e., that the K^+ permeability of the resting membrane is low. The resting membrane potential of the sperm was estimated about −50 mV in normal ASW with $[K^+]_{ex}$ of 9 mM (Fig. 5).

Fluorescence intensity decreased when K^+ ionophore Val (Fig. 4B) or SAAF (Fig. 4C) was added, suggesting that Val and SAAF increased K^+ permeability of the sperm plasma membrane and resulted in its hyperpolarization. Subsequent additions of KCl to the external solution caused depolarization of the cells (Figs. 4B and 4C, arrows; cf. Fig. 4A), which means that the sperm plasma membrane is now permeable to K^+. The degree of hyperpolarization in response to Val and SAAF was dependent on $[K^+]_{ex}$, and both hyperpolarizations reached the same level (−100 mV) in ASW (Fig. 5), suggesting that the SAAF-induced permeability of the plasma membrane is comparable to that induced by Val.

When the change of intracellular potassium concentration was monitored by the change of fluorescence in the PBFI-loaded sperm, the fluorescence was decreased by the addition of SAAF (Fig. 6A). This suggests the occurrence of K^+ efflux during the membrane hyperpolarization (see Fig. 4C) at the initiation of sperm motility by SAAF (Yoshida *et al.*, 1994). Subsequent additions of KCl did not induce the increase in $[K^+]_{in}$, although they induced the depolarization of the membrane (Fig. 4).

pH_{in} of the sperm was measured to be 6.94 ± 0.1 (n = 16) by BCECF-AM. Then $[K^+]_{in}$ of the immotile sperm was calculated to be 560 ± 40 mM (n = 14) as described under Materials and Methods.

Cation selectivity of the SAAF-induced permeability of

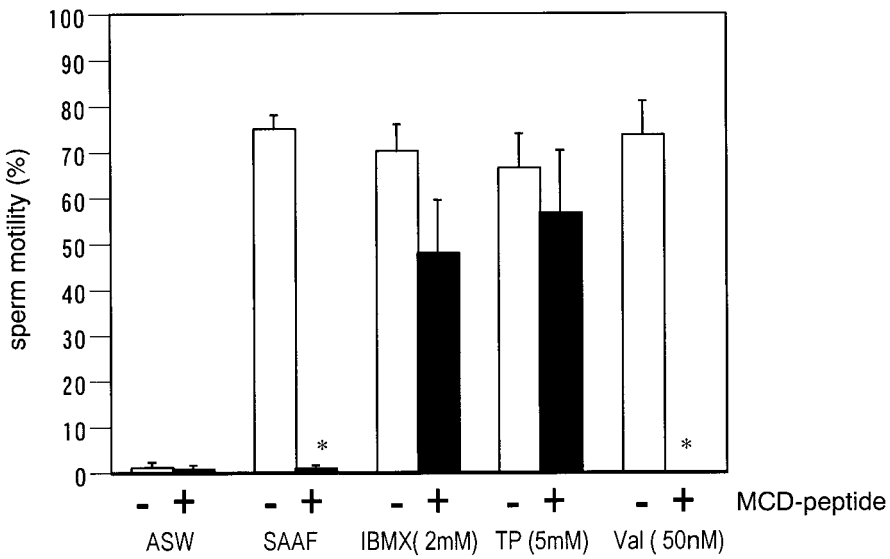

FIG. 3. Effect of MCD peptide on the activation of sperm motility. In normal ASW, SAAF, Val, IBMX, and TP all induced sperm motility (open column). In the presence of the K^+ channel blocker MCD peptide (filled column), SAAF- and Val-induced sperm motility was completely inhibited (*), but that induced by the two phosphodiesterase inhibitors (IBMX, TP) was not inhibited significantly. Data represent means ± SD (n = 5).

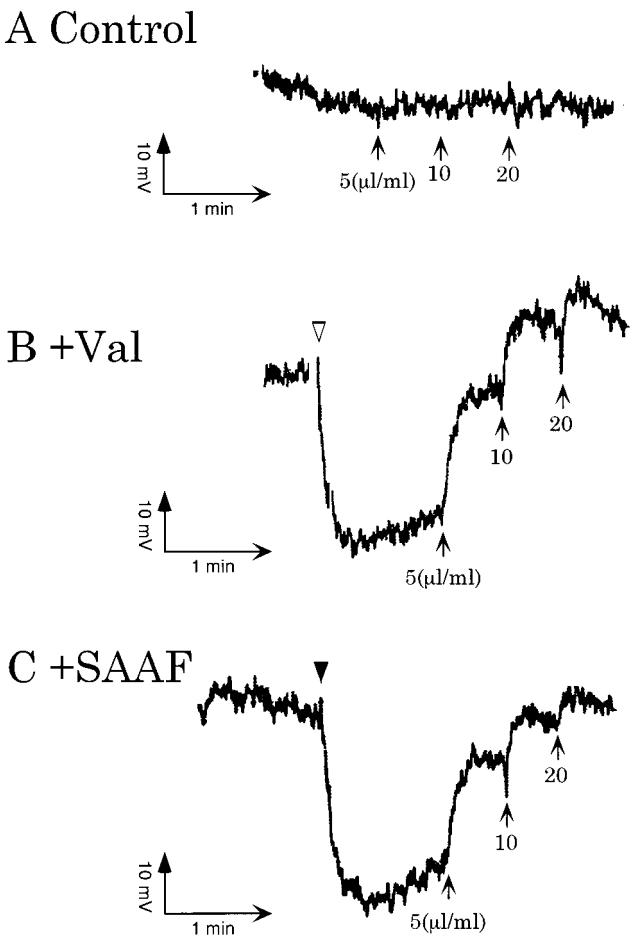

FIG. 4. Effects of SAAF and valinomycin on the membrane potential. Sperm was diluted with ASW (9 mM K^+) containing 0.5 μM $DisC_3(5)$, and the fluorescence was measured and converted to the V_m as described under Materials and Methods. Downward indicates hyperpolarization. In the absence of SAAF or Val, addition of 5, 10, and 20 μl of 2 M K^+ (arrows) to the sperm suspension did not induce any fluorescence change (A). Addition of Val (open arrowhead in B) or SAAF (filled arrowhead in C) decreased the fluorescence, suggesting an increase in K^+ permeability and hyperpolarization of the sperm plasma membrane. Subsequent addition of 5, 10, and 20 μl of 2 M K^+ (arrows) caused membrane depolarizations (B, C).

sperm plasma membrane was $K^+ \geqq Rb^+ \gg Cs^+ > Li^+$ (Fig. 7).

MCD peptide partially inhibited SAAF-induced hyperpolarization of the sperm plasma membrane. When SAAF was added to the sperm suspension in the control ASW, it induced hyperpolarization of the sperm, and further addition of Val did not result in a further hyperpolarization. Subsequent addition of K^+ caused depolarization (Fig. 8A, see also Fig. 4). When the sperm were diluted into a solution containing 10 μM MCD peptide, the addition of SAAF and then Val resulted in less hyperpolarization (Fig. 8B). Depolarizations of the plasma membrane by the subsequent additions of K^+ were much smaller in the presence of MCD peptide (Fig. 8B).

SAAF- and Valinomycin-Induced cAMP Synthesis

The sperm diluted in normal ASW were almost immotile, and the concentration of intracellular cAMP was as low as 0.5 nmol/mg protein (Fig. 9A). When SAAF was added to the immotile sperm, the cAMP increased to about 2.4 ± 0.5 nmol/mg protein within 5 s, and the sperm became motile. The cAMP level decreased during the next 15 s and remained low (less than 1 nmol/mg protein). Val also activated the sperm motility (see Fig. 2) and induced a sustained increase in cAMP up to 1.4 ± 0.4 nmol/mg protein within 5 s. IBMX (2 mM) also induced sperm motility and increased cAMP to about 7.5 ± 1.0 nmol/mg protein. IBMX at 200 μM neither increased cAMP nor induced sperm motility.

IBMX did not hyperpolarize the sperm cells (not shown). Figure 9B shows that in the presence of 2 mM IBMX, SAAF or Val induced a further increase in the cAMP level. This suggests that both SAAF and Val increase the cAMP level through the activation of adenylyl cyclase and that the

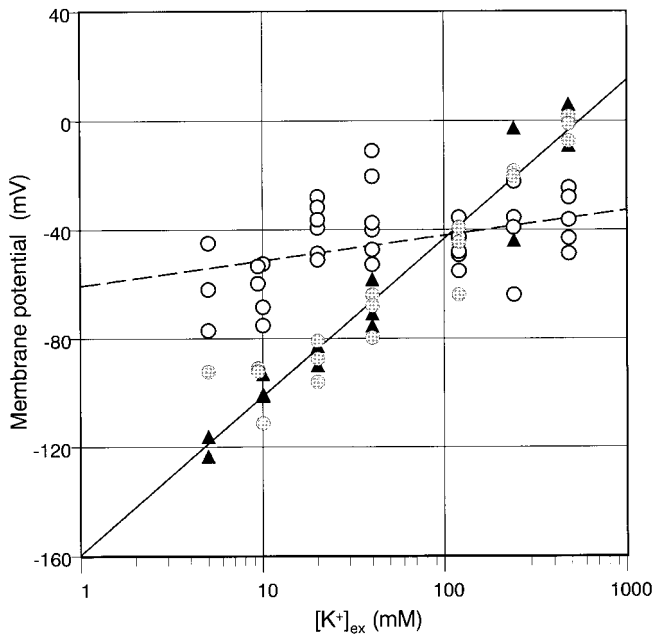

FIG. 5. Summary of relationships between $[K^+]_{ex}$ and the V_m as described in Fig. 4. The open circles indicate the relationship in the absence of SAAF or Val, and the filled circles and the filled triangles indicate that in the presence of SAAF and Val, respectively. The dotted and solid lines indicate the linear regression lines in membrane potentials in the absence of SAAF or Val and in the presence of Val, respectively. The linear regression lines could be expressed as $V_m = 4.0452 \ln([K^+]_{ex}) - 60.55$ and $V_m = 25.26 \ln([K^+]_{ex}) - 159.84$, respectively. Sperm from two *Ciona* were used.

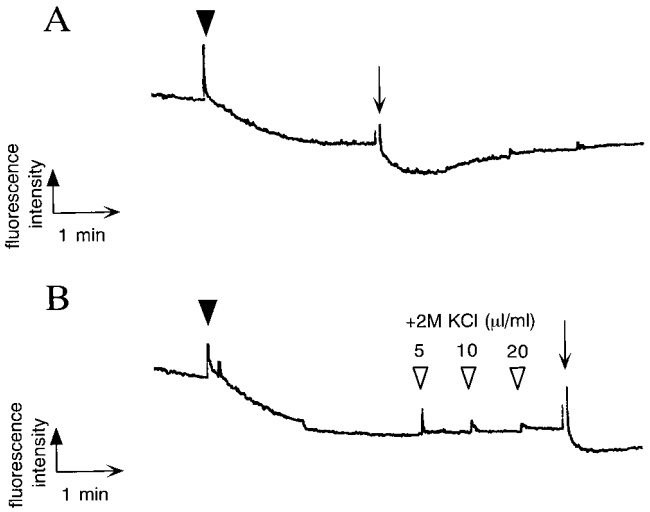

FIG. 6. Changes in intracellular K^+ concentration during the activation of sperm motility. Sperm was loaded with PBFI-AM, a fluorescent potassium indicator, and $[K^+]_{in}$ was monitored as described under Materials and Methods. Downward indicates decrease in $[K^+]_{in}$. (A) Addition of SAAF (filled arrowhead) induced a decrease in $[K^+]_{in}$, i.e., K^+ efflux. The further decrease in fluorescence induced by the addition of digitonin (arrow) indicates that $[K^+]_{in}$ is higher than $[K^+]_{ex}$. (B) After addition of SAAF (filled arrowhead), subsequent addition of potassium to external solution did not increase $[K^+]_{in}$, although it depolarized the membrane (Fig. 5). K^+ efflux after the digitonin treatment (arrow) indicates that $[K^+]_{in}$ is higher than the final $[K^+]_{ex}$ of 80 mM.

activity of the enzyme is facilitated by the hyperpolarization.

The MCD peptide inhibited the SAAF-induced cAMP elevation, while it did not do so significantly in the Val or IBMX-treated sperm (Fig. 10).

Ion Concentrations of Seminal Plasma and Body Fluid

Ion concentrations and pH of the seminal plasma, body fluid, and natural seawater were measured and are listed in Table 1. The Ca^{2+}, K^+, Mg^{2+}, and Cl^- concentrations of the seminal plasma, the body fluid, and the natural seawater (NSW) were almost the same. The Na^+ concentration of body fluid was slightly higher than that of the seminal plasma and NSW (495 ± 40, 445 ± 43, and 450 mM, respectively). The pH of body fluid varied between 6.6 and 7.0 (n = 8), which was significantly lower than that of the NSW (pH 8.3–8.4).

DISCUSSION

The sperm-activating and -attracting factor (SAAF), which was discovered in the ascidians *C. intestinalis* and *C. savignyi*, is considered a unique molecule that controls the two phenomena, sperm activation and chemotaxis (Yoshida *et al.*, 1994). The sperm of these species exhibit low motility in seawater. When SAAF is released from the vegetal pole of the spawned egg and reaches the sperm, it stimulates both Ca^{2+} influx and a transient cAMP elevation (Yoshida *et al.*, 1994; Fig. 9), leading to the activation of sperm motility. Calcium is required for the induction of sperm chemotaxis by SAAF, which leads them toward the vegetal pole of the egg to establish fertilization (Yoshida *et al.*, 1994).

In the present study, we showed that SAAF increases K^+ permeability of sperm, resulting in hyperpolarization (Figs. 4 and 5) and the activation of sperm motility in *C. intestinalis*. The K^+ ionophore Val hyperpolarized the membrane potential by increasing K^+ permeability (Fig. 4) and mimicked the inductive action of SAAF on sperm motility (Fig. 1). The sperm motility induced by SAAF as well as by Val was inhibited by high $[K^+]_{ex}$ (Fig. 2). These results suggest that the hyperpolarization of the sperm induced by an increase in K^+ permeability and subsequent K^+ efflux is important for the activation of sperm motility.

K^+ permeability mainly contributes to the resting potential of many kinds of cells (Hodgkin and Horowicz, 1960). It has also been reported that the changes of membrane potential and of K^+ permeability regulate sperm functions. With regard to the acrosome reaction, the membrane potential of sea urchin sperm in seawater is almost unresponsive to the addition of KCl in seawater, indicating low resting K^+ permeability of sperm (Babcock *et al.*, 1992, Beltran *et al.*, 1996). The egg decapeptide, speract, increases K^+ permeability by increasing cGMP (Cook and Babcock, 1993a) and thus induces hyperpolarization of the sperm membrane (Babcock *et al.*, 1992; Reyoud *et al.*, 1993). Hyperpolarization followed by depolarization (González-Martínez *et al.*, 1992) is necessary for the elevation of cAMP (Cook and Babcock, 1993b; Beltran *et al.*, 1996) and subsequent Ca^{2+} influx, respectively, resulting in the acrosome reaction. Sperm motility is decreased by TEA^+, a delayed rectifier K^+ channel blocker (Tanimoto and Morisawa, 1988), and the membrane potential of sperm is sensitive to the transmembrane concentration gradient of K^+ (Gatti *et al.*, 1990) in the rainbow trout. In addition, the sperm motility, which is inhibited in the presence of high $[K^+]_{ex}$ (Morisawa and Suzuki, 1980; Morisawa *et al.*, 1983), is rescued in response to the hyperpolarization of sperm by valinomycin or by divalent cation in trout sperm (Boitano and Omoto, 1991). A voltage-dependent K^+ channel blocker, 4-AP, reversibly blocks hyperpolarization-induced motility of common carp sperm (Krasznai *et al.*, 1995; Emri *et al.*, 1998), suggesting the contribution of K^+ permeability and membrane potential changes of the plasma membrane in the regulation of sperm motility.

We showed here that the membrane potential and the intracellular K^+ concentration of the immotile sperm without SAAF was calculated to be about −50 mV (Fig. 5) and 560 ± 40 mM, respectively, and that the membrane potential was hyperpolarized to about −100 mV (Fig. 5) by K^+

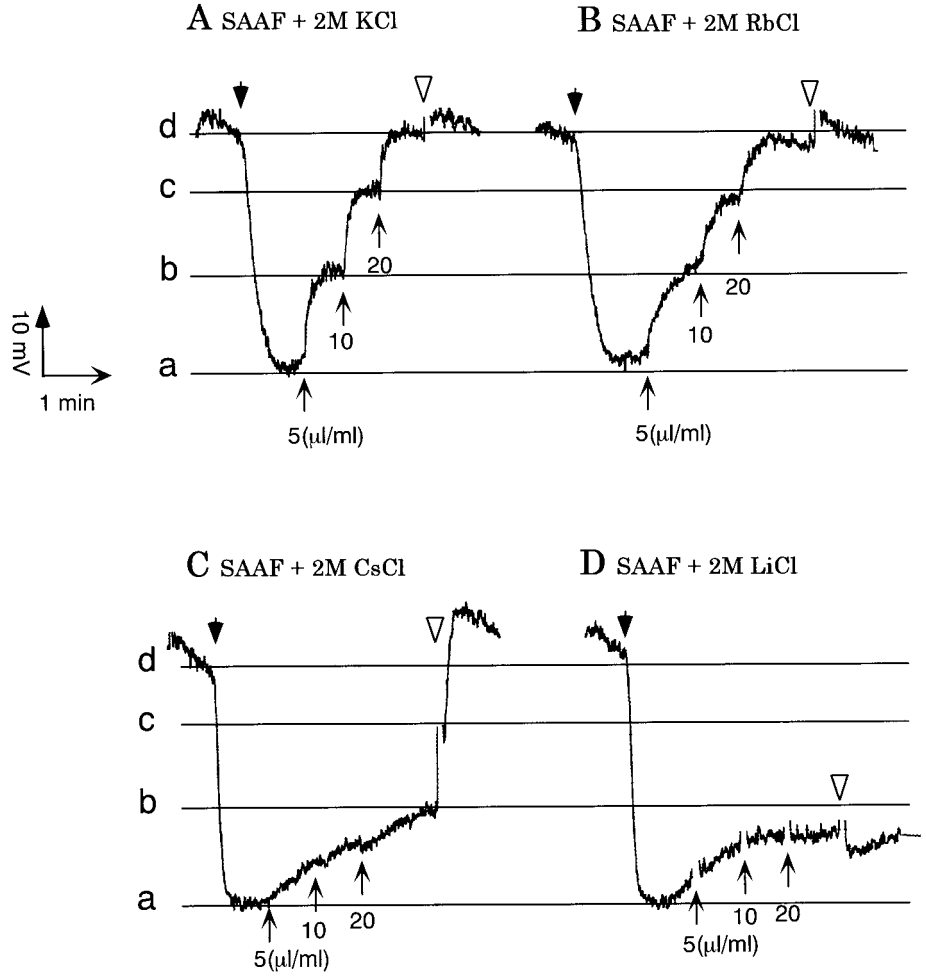

FIG. 7. Cation selectivity of permeability in SAAF-treated plasma membrane. Fluorescence was monitored by the same method as described for Fig. 4. SAAF (filled arrowhead) increased K^+ permeability, resulting in hyperpolarization of the plasma membrane. Depolarization was induced by subsequent additions of K^+ (A). Horizontal line (a) indicates the base membrane potentials of the SAAF-treated sperm in ASW (10 mM K^+), and the lines (b), (c), and (d) indicate the membrane potentials when 5, 10, and 20 μl of 2 M KCl were added to SAAF-treated sperm (final $[K^+]_{ex}$ 20, 40, and 80 mM). The order of permeability of the plasma membrane was K^+ (A) $\geqq$ Rb^+ (B) $\gg$ Cs^+ (C) $>$ Li^+ (D).

efflux (Fig. 6) when the sperm motility was activated by SAAF. Results of experiments using MCD peptide clearly showed that activation of K^+ channels by SAAF hyperpolarizes the plasma membrane (Fig. 8) and activates sperm motility in *Ciona*.

How does the hyperpolarization of the plasma membrane by SAAF induce sperm activation? It is well known that the forward and backward movements of *Paramecium* are controlled by the second messengers Ca^{2+} and cAMP (Nakaoka and Ooi, 1985). Schultz *et al.* (1992) further suggested that the adenylyl cyclase of this species shows properties of a voltage-independent K^+ channel and that a hyperpolarization-activated K^+ efflux appears to directly regulate adenylyl cyclase activity, resulting in cAMP increase in the cell and regulation of ciliary movement in the unicellular organism. In the sperm of the ram, dog, and human, adenylyl cyclase is also known to be a membrane-bound protein (Hildebrandt *et al.*, 1985; Tang and Gilman, 1992), and the activity of the enzyme can be regulated by the change of membrane potential. The enzyme is also present in the sea urchin sperm as a membrane-bound protein with affinity to calmodulin (Bookbinder *et al.*, 1990). The enzyme is activated by speract-induced membrane hyperpolarization and synthesizes cAMP, leading to the acrosome reaction in the presence of Ca^{2+} at fertilization (Beltran *et al.*, 1996). Cyclic AMP synthesis caused by hyperpolarization of the sperm also induces the sperm capacitation in mammals (Zeng *et al.*, 1995). Similarly, in *Ciona* sperm (present study), Val and SAAF, which induced hyperpolarization of the sperm, also increased cAMP (Fig. 9). The

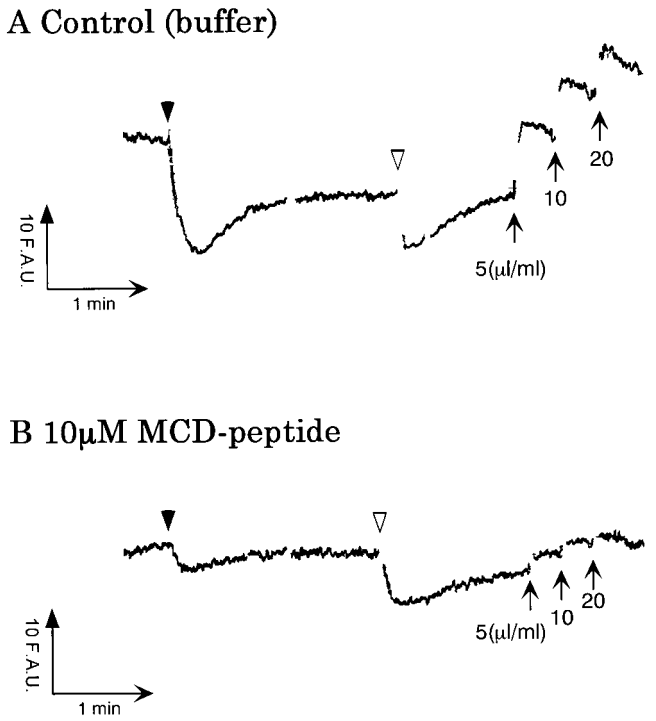

FIG. 8. Effects of MCD peptide on the membrane potential of the sperm. Fluorescence was monitored by the same method as described for Fig. 4. (A) Addition of SAAF (filled arrowhead) hyperpolarized the plasma membrane, and subsequent addition of Val (open arrowhead) hyperpolarized the sperm to a similar level. (B) Hyperpolarizations were partially inhibited when SAAF and Val were added in the presence of 10 μM MCD peptide. Depolarizations of the plasma membrane by the subsequent additions of K^+ (arrows) were decreased in the presence of MCD peptide (B).

application of IBMX or theophylline, which are well-known blockers of the phosphodiesterase, resulted in the elevation of the cAMP level (Fig. 9; Yoshida *et al.*, 1994), causing the initiation of *Ciona* sperm motility (Fig. 3). This suggests that there exists a basal level of cAMP synthesis. In the presence of 2 mM IBMX, which induced sperm motility, SAAF or Val induced a further increase in cAMP (Fig. 9), suggesting that SAAF and Val act not by inhibiting phosphodiesterase but by activating adenylyl cyclase through hyperpolarization of sperm. MCD peptide inhibited SAAF-induced phenomena at the activation of sperm by blocking K^+ channels, hyperpolarization, cAMP synthesis, and sperm motility. However, the blocker did not inhibit cAMP synthesis by Val significantly (Fig. 10).

Gauss *et al.* (1998) cloned, functionally expressed, and characterized SPIH, a weakly K^+-selective channel that is present in the flagellum of sea urchin sperm. SPIH is gated by both hyperpolarization and cAMP and is blocked by Cs^+. Because K^+ permeability was not increased by IBMX (not shown), which caused cAMP elevation (Fig. 9), and was not affected by 40 mM Cs^+ in the bath solution (not shown), it is considered that the homolog of SPIH is not present in *Ciona* sperm, or that, if present, it is not a major component of the increased K^+ permeability which underlies the action of SAAF.

Ca^{2+} is also known to be the second messenger that participates in the activation of sperm motility and chemotaxis in *Ciona* sperm (Yoshida *et al.*, 1994). In the present study, Val caused the initiation of sperm motility in CaF-ASW, while SAAF caused neither motility nor chemotaxis

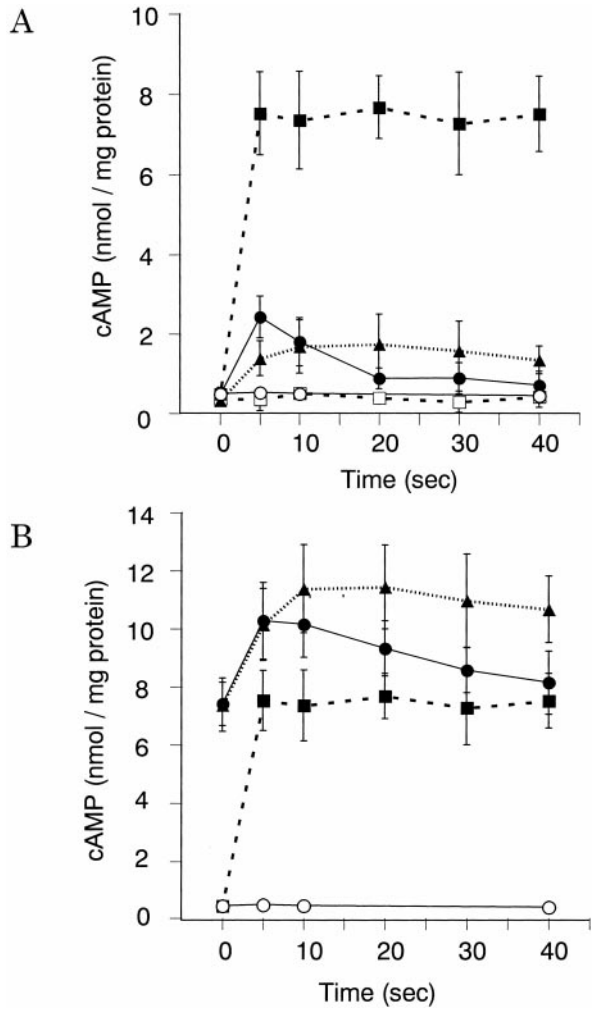

FIG. 9. Changes in cAMP level at the activation of sperm motility by SAAF, Val, or IBMX. (A) cAMP level of sperm was not changed after the addition of mixed DMSO and DW (control, open circle). The cAMP concentration of the sperm reached a peak in 5 s after the addition of SAAF (filled circle), then it gradually decreased. 100 nM Val (filled triangle) gradually increased the cAMP concentration, which reached a plateau after 10 s. Two millimolar IBMX (filled square) maintained a high cAMP level continuously at about 7.5 nmol/mg protein. (B) After Incubation with 2 mM IBMX (40 s, filled square), SAAF (filled circle) or Val (filled triangle) induced further increase in cAMP. Data represent means ± SD (n = 3).

in CaFASW. In our preliminary studies using fluorescent dye, fluo-4, a small vesicle which accumulated Ca^{2+} was found in the space between the nucleus and the mitochondria, similar to the one reported by Georges (1969). Furthermore, SAAF treatment induced Ca^{2+} release from this calcium store in ASW, while it did not do so in CaFASW. Val treatment induced the Ca^{2+} release in ASW and CaFASW, although the mechanism of this Ca^{2+} release is unknown at present. This may be the reason why Val induced sperm motility in CaFASW. And it might be suggested that Val failed to induce the Ca^{2+} release and sperm motility because of insufficient hyperpolarization in the presence of MCD peptide.

Based on the present study, we propose a working hypothesis in regard to the activation of *Ciona* sperm motility as follows. The SAAF induces membrane hyperpolarization by increasing the K^+ permeability. This hyperpolarization triggers cAMP production through the activation of adenylyl cyclase, which may be a transmembrane protein and may be regulated by membrane potential. The subsequent increase of cAMP stimulates the cAMP-dependent protein kinases, which then phosphorylate certain protein(s) (Dey and Brokaw, 1991) and activate sperm motility.

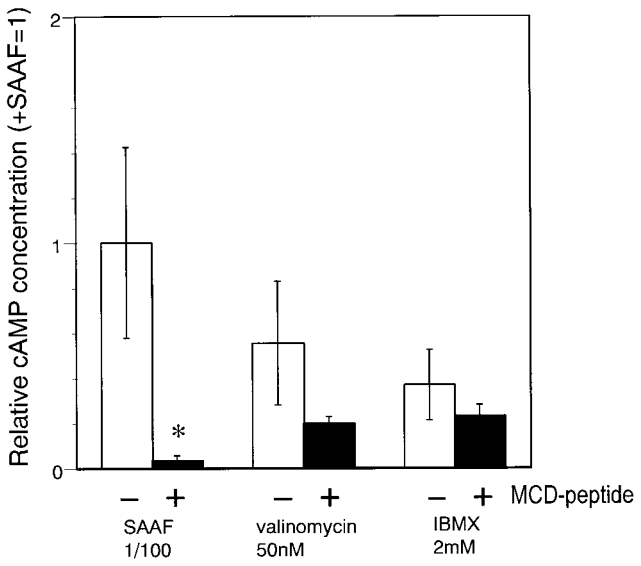

FIG. 10. Effects of MCD peptide on the cAMP synthesis induced by SAAF, Val, or IBMX. cAMP levels of SAAF, Val (50 nM), or IBMX (2 mM)-treated sperm were measured at 5 seconds as described under Materials and Methods in the absence (open column) or presence (filled column) of 10 μM MCD peptide. The MCD peptide significantly (*) inhibited the SAAF-induced cAMP elevation, while it did not do so in the Val- or IBMX-treated sperm. Sperm motility by SAAF or Val was completely inhibited in the presence of MCD peptide. Data represent means ± SD (n = 5).

TABLE 1

Ion Concentration and pH of Seminal Plasma, Body Fluid of *Ciona intestinalis*, and Natural Seawater at Misaki

(mM)	Seminal plasma[a]	Body fluid[b]	Seawater in Misaki
Na^+	445 ± 42.91	495.83 ± 39.33	450
Ca^{2+}	8.44 ± 0.75	9.58 ± 0.66	10.15
K^+	10.18 ± 0.88	10.77 ± 1.07	10.7
Mg^{2+}	48.35 ± 4.37	51.4 ± 1.77	48.7
Cl^-	555.75 ± 23.77	545 ± 19.09	537
pH	ND	6.6–7.0	8.3–8.4

[a] Mean ± SD (n = 8).
[b] Mean ± SD (n = 6).

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